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Rapid formation of inositol 1,3,4,5-tetrakisphosphate following muscarinic receptor stimulation of rat cerebral cortical slices

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Carbachol stimulation of muscarinic receptors in rat cortical slices prelabelled with *myo*-[2-^{3}H]inositol caused the rapid formation of a novel inositol polyphosphate. Evidence derived from its chromatographic behaviour, and from the structure of the products formed in partial dephosphorylation experiments, suggests that it is probably D-*myo*-inositol 1,3,4,5-tetrakisphosphate. An enzyme in human red cell membranes specifically removes the 5-phosphate from it to form inositol 1,3,4-trisphosphate. It is suggested that inositol 1,3,4,5-tetrakisphosphate is likely to be a second messenger, and that it is the precursor of inositol 1,3,4-trisphosphate and possibly of inositol 1,4,5-trisphosphate.

INTRODUCTION

Considerable progress has been made recently in our understanding of the function of stimulated inositide turnover, in particular with the identification of PtdIns(4,5)P_2 as a primary target for receptor-stimulated hydrolysis (Michell *et al.*, 1981; Berridge, 1983, 1984), and the subsequent demonstration that Ins(1,4,5)P_3 is the likely intracellular message for calcium mobilization (Streb *et al.*, 1983; Berridge & Irvine, 1984). Measurement of Ins(1,4,5)P_3 in stimulated tissues has been complicated by the finding that another inositol trisphosphate, Ins(1,3,4)P_3, is formed which has entirely different metabolic kinetics (Irvine *et al.*, 1984*a*, 1985; Burgess *et al.*, 1985). The source and function of Ins(1,3,4)P_3 have so far remained unidentified.

Stimulation of a variety of neurotransmitter receptors in brain slices, in common with receptors in many other tissues, causes formation of inositol phosphates (Brown *et al.*, 1984; Daum *et al.*, 1984; Gonzalez & Crews, 1984). During a more detailed study of muscarinic stimulation in cortical slices, we are able not only to identify the two isomers of InsP_3, but also preliminary experiments suggested a more polar inositol phosphate was being rapidly formed. Here we present evidence that this is an entirely novel inositol phosphate, Ins(1,3,4,5)P_4.

MATERIALS AND METHODS

Tissue preparation

Cerebral cortical slices (350 μm × 350 μm) were prepared from male Sprague–Dawley rats and preincubated for 60 min in Krebs–Henseleit buffer as previously described (Brown *et al.*, 1984). Aliquots (50 μl) of packed slices (1–2 mg of protein) were then incubated at 37 °C in flat-bottomed vials with *myo*-[2-^{3}H]inositol (Amersham International), 5 μCi/vial (approx. 1.5 μM) for 60 min in a total volume of 300 μl. Reactions were terminated with 300 μl of 1 M-trichloroacetic acid, and tissue was sedimented by centrifugation. The acid extracts were washed with 5 × 2 vol. of water-saturated diethyl ether and adjusted to pH 7–8 with $NaHCO_3$. In some experiments for preparative purposes these procedures were scaled up 15-fold.

Separation of radiolabelled inositol phosphates

For preparative purposes, for preliminary examinations and for partial dephosphorylation experiments, the inositol phosphates were separated on Dowex-1 anion exchange columns (8% cross-linked, mesh size 200–400, formate form; Berridge *et al.*, 1983). A clear separation of InsP_3 and InsP_4 was achieved by elution of the former from columns (2 cm × 0.5 cm) with 10 ml of 0.1 M-formic acid/0.8 M-ammonium formate, followed by elution of the latter with 10 ml of 0.1 M-formic acid/1.0 M-ammonium formate. This separation method was repeatedly checked by the various analytical procedures described below, and by using standards of [^{3}H]InsP_4 from stimulated brain slices and [^{32}P]Ins(1,4,5)P_3, and it provides a convenient way of separating the two inositol polyphosphates after prior elution of inositol, Ins$P$ and InsP_2 (Berridge *et al.*, 1983).

For a more detailed analysis, including separation of the InsP_3 isomers, a modification of an h.p.l.c. ion-exchange system (Irvine *et al.*, 1985) was used. The eluant was increased linearly from 0 to 0.75 M-ammonium formate buffered to pH 3.7 with orthophosphoric acid over 5 min to elute lower inositol phosphates, and then the buffer held at this concentration for 2 min. Ins(1,3,4)P_3 and Ins(1,4,5)P_3 were then eluted (distinct from each other) by a linear increase over 6 min to 1.0 M-ammonium formate/phosphoric acid, pH 3.7 (cf. Irvine *et al.*, 1985). After a further 5 min at this strength, the buffer was then increased over 10 min to a final concentration of 1.7 M-ammonium formate/phosphoric acid, pH 3.7, and held there for 6 min before returning to water. InsP_4 was found to be eluted at about 1.5 M-ammonium formate/phosphoric acid, pH 3.7.

Abbreviations used: Ins$P$, InsP_2, InsP_3, InsP_4, InsP_5 and InsP_6, inositol mono-, bis-, tris-, tetrakis-, pentakis- and hexakis-phosphates respectively, with assignment of phosphate locants where appropriate [e.g. Ins(1,3,4)P_3]; PtdIns, PtdIns$P$, PtdInsP_2 and PtdInsP_3, phosphatidylinositol and its mono-, bis- and tris-phosphorylated derivatives.

Standards

[^{32}P]Ins(1,4,5)P_3 was prepared as in Downes *et al.* (1982). [^{3}H]InsP_4 was prepared in bulk from carbachol-stimulated brain slices by Dowex ion-exchange chromatography (as above), spiked with 200 nmol of non-radioactive Ins(1,4,5)P_3 to aid desalting, and then desalted by elution with 1.5 M-LiCl from a Dowex (chloride) column followed by removal of the LiCl with ethanol (Tomlinson & Ballou, 1961; Burgess *et al.*, 1984). Its purity was checked by h.p.l.c. and ionophoresis.

A mixture of InsP_5 and InsP_4 was prepared by partial dephosphorylation of InsP_6 (phytic acid) at pH 4, exactly as in Desjobert & Petek (1956). Non-radiolabelled Ins(1,4,5)P_3 was prepared as in Irvine *et al.* (1984*b*).

Alditols were obtained as in Irvine *et al.* (1984*a*).

Separation of inositol phosphates

Separation of InsP_6, InsP_5, InsP_4 and InsP_3 was achieved by paper chromatography (Desjobert & Petek, 1956) or by ionophoresis in 0.1 M-sodium oxalate, pH 1.5 (Seiffert & Agranoff, 1965).

Identification of alditols after periodate degradation of inositol phosphates

Periodate oxidation, borohydride reduction and alkaline phosphatase dephosphorylation of radiolabelled inositol phosphates was exactly as in Irvine *et al.* (1984*a*) except that only 0.25 ml of 0.1 M-sodium periodate and 5 mg of sodium borohydride were used. In all experiments > 80% recovery of a radiolabelled alditol from the original inositol tetrakis- or tris-phosphate was achieved. Identification of alditols was by co-chromatography with internal standards in two entirely different separation systems, ionophoresis in 0.1 M-NaOH, and chromatography in ethyl acetate/pyridine/boric acid-saturated water (12:5:4, by vol.) (Irvine *et al.*, 1984*a*). In all instances two or three separate runs in each system with all probable standards were employed so that the identification of any ^{3}H-labelled alditols, although indirect, was unambiguous.

Partial dephosphorylation of InsP_4

This was achieved in two ways. Firstly, by alkaline phosphatase at pH 9.0. Trial-and-error experiments were performed using various dilutions and incubation times with calf intestine alkaline phosphatase (Boehringer Mannheim), separating InsP_4 and InsP_3 by Dowex anion-exchange chromatography (see above). Once conditions were optimized (25–30% conversion of InsP_4 to InsP_3) a bulk preparation was made; alkaline phosphatase (calf intestine, grade II; Boehringer Mannheim) was diluted 50-fold in 0.1% bovine serum albumin, and 50 μl of this diluate was incubated for 15 min at pH 9.0 (Tris/maleate buffer) with 2 mM-Mg^{2+} in a final volume of 2 ml. The InsP_3 formed was separated by ion exchange and desalted as above, before periodate, borohydride and alkaline phosphatase treatment, or analysis by ionophoresis.

Secondly, human red blood cell membranes (Downes *et al.*, 1982; 2 mg of protein) were incubated at pH 7.5 in a final volume of 2 ml with 2 mM-Mg^{2+} as described in Irvine *et al.* (1984*a*), and they were found to convert the InsP_4 almost quantitatively (> 80% in 1 h) to InsP_3. This InsP_3 was separated and desalted as described above before analysis by h.p.l.c. or periodate, borohydride and alkaline phosphatase treatment (as above).

RESULTS

General properties of the polar inositol polyphosphate

The observation that a more polar inositol polyphosphate could easily be separated by ion-exchange chromatography from the inositol trisphosphates (see the Materials and methods section) not only gave us a means of purifying it in large quantities, but in itself suggested this polar inositol polyphosphate may not be an inositol trisphosphate. This suggestion was strengthened by the findings (*a*) that to elute the polar inositol polyphosphate from a Partisil SAX-10 h.p.l.c. column, an elution medium of about 50% greater strength than that used for inositol trisphosphates was required and (*b*) that partial dephosphorylation of the polar inositol polyphosphate by alkaline phosphatase or human red cell membranes (see below) yielded compounds which by their ionophoretic and chromatographic behaviour are inositol trisphosphates.

That this more polar inositol polyphosphate is a tetrakisphosphate was confirmed by running it in two separation procedures which distinctly separate InsP_3, InsP_4 and InsP_5 (see the Materials and methods section); the separations were carried out with internal InsP_6, InsP_5 and InsP_3 markers and also with internal [^{32}P]Ins(1,4,5)P_3. In both ionophoresis and paper chromatography the novel compound co-chromatographed with InsP_4. A potential difficulty in making this identity certain emerged in the observation that in addition to the strong InsP_5, InsP_4 and InsP_3 spots visible on ionophoresis of a phytic acid partial hydrolysate, minor spots are also evident caused by some separation of isomers (Seiffert & Agranoff, 1965); the radiolabelled inositol phosphate in question migrated so that it overlapped mostly with the strong InsP_4 spot, but partly with a minor spot migrating just behind whose identity was not certain. To ensure it really was migrating with InsP_4, we performed some experiments in which the length of the ionophoresis run was extended so that InsP_6 and InsP_5 ran off the end of the paper; in these experiments InsP_4 isomers ran as a distinct group with a 4 cm gap clearly separating them from the InsP_3 isomers. The radiolabelled novel inositol phosphate still migrated as InsP_4 under these conditions, mostly overlapping with the major InsP_4 spot, and partly with a minor spot migrating just behind. Paper chromatography gave no hint of InsP_4 isomer separation in our hands, and the radioactive compound co-migrated exactly with the InsP_4 marker.

If the putative InsP_4 was treated extensively with alkaline phosphate, the only radiolabelled product recovered was inositol, as judged by the two separation methods given in the Materials and methods section and also chromatography in propan-1-ol/ethyl acetate/water (24:13:7, by vol.). This evidence shows that our novel compound is an inositol tetrakisphosphate, and, notwithstanding the remote possibility of an isomerization of the radiolabelled *myo*-inositol (see Irvine *et al.*, 1984*a*), we assume that it is a *myo*-inositol tetrakisphosphate.

Metabolism of InsP_4 in stimulated brain slices

Fig. 1 shows a typical result of an experiment to measure the rates of appearance of Ins(1,4,5)P_3, Ins(1,3,4)P_3 and InsP_4 in cortical slices stimulated with a 10^{-3} M concentration of the cholinergic agonist carbachol. Stimulation of each of these polyphosphates was suppressed if slices were preincubated with the

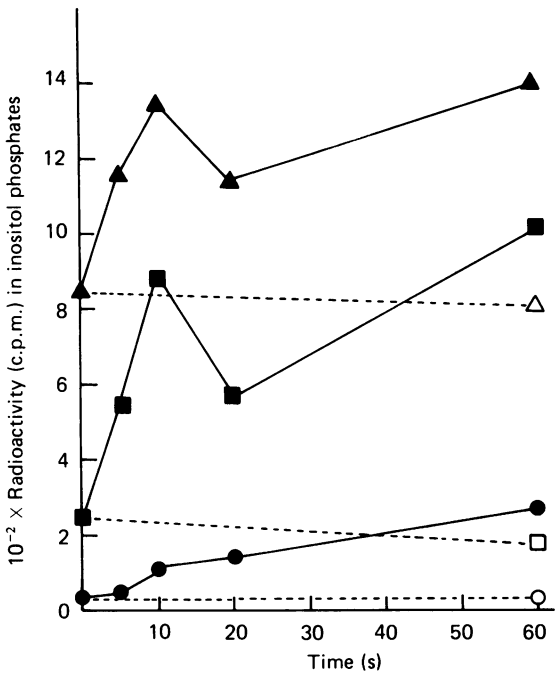

Fig. 1. Time course of inositol tris- and tetrakisphosphate formation in carbachol-stimulated brain slices

Brain slices labelled with *myo*-[2-^{3}H]inositol were stimulated with carbachol (10^{-3} M) for various times as described in the Materials and methods section. InsP_3 isomers and InsP_4 were separated by h.p.l.c. Each data point is the result of the pooling of three separate incubations [to ensure sufficient radioactivity in Ins(1,3,4)P_3]. The data are from one experiment, though very similar data were obtained in another experiment analysed by h.p.l.c., and in two further experiments using Dowex ion-exchange chromatography (which separates InsP_3 and InsP_4, but not the InsP_3 isomers). ○, △, □, controls; ●, ▲, ■, carbachol-treated; ○, ●, Ins(1,3,4)P_3; △, ▲, Ins(1,4,5)P_3; □, ■, Ins(1,3,4,5)P_4.

muscarinic antagonist, atropine (10^{-5} M). It is apparent that Ins(1,4,5)P_3 and InsP_4 are both produced rapidly (within 5 s) but that there may be a lag before Ins(1,3,4)P_3 rises, as in other tissues (Irvine *et al.*, 1985; Burgess *et al.*, 1985), although, because of the low radioactivity in Ins(1,3,4)P_3, we cannot be sure of this. Also, the specific activities of InsP_3 and InsP_4 are not known, so we cannot compare absolute rates of synthesis. The essential point of Fig. 1 is that [^{3}H]InsP_4 is rapidly produced after carbachol stimulation of cortical slices.

Structure of InsP_4

In investigating the distribution of phosphates around the inositol ring (and we shall assume a *myo*-inositol ring for the arguments in this section) we have used the same methods as previously (Irvine *et al.*, 1984*a*). These are, in turn, adapted from the techniques of Ballou and co-workers (Grado & Ballou, 1961; Tomlinson & Ballou, 1961). Periodate oxidation splits the bond between two carbon atoms bearing hydroxyls, whereas the presence of a phosphate group on one or both of the carbon atoms protects the bond from attack. So, periodate treatment of an inositol polyphosphate, followed by reduction and dephosphorylation and identification of the resulting alditol, yields information about the distribution of phosphates in the inositol ring.

When [^{3}H]InsP_4 from brain slices was submitted to this periodate, borohydride and alkaline phosphatase treatment, the predominant (> 90%) product was inositol, even if the periodate treatment was allowed to proceed for 5 days at pH 6.5. This means that the InsP_4 has no vicinal hydroxyls. Thus the phosphates must be distributed either as two pairs, or as a single with a group of three. These two alternatives can be distinguished, and the structure of the InsP_4 explored, by partial dephosphorylation to InsP_3 using alkaline phosphatase. Although essentially non-specific in its phosphatase activity on inositol phosphates, alkaline phosphatase greatly favours a solitary phosphate group over a phosphate group adjacent to other phosphates (Tomlinson & Ballou, 1961). Thus conversion of the InsP_4 to InsP_3, followed by periodate, borohydride and alkaline phosphatase treatment, should yield a complex mixture of hexitols if the InsP_4 phosphates are paired, but only one product, a pentitol, if there is a single phosphate with a group of three. The latter was what we found; xylitol was the only detectable (> 90%) labelled product after periodate, borohydride and alkaline phosphatase treatment of the resulting InsP_3. We carried out the ionophoretic separation of alditols in 0.1 M-NaOH (see Irvine *et al.*, 1984*a*) using both long (4.5 h) runs which clearly separate xylitol from all alditols except arabitol, and short (2 h) runs to ensure that no fast-migrating compounds (e.g. tetritols) were formed. Arabitol and xylitol were then distinctly separated by paper chromatography (Grado & Ballou, 1961; Irvine *et al.*, 1984*a*) to confirm the identity of the alditol as xylitol. These results indicate a D- or L-(3,4,5) grouping of phosphates in the original InsP_4. [We should note in passing that another InsP_3, Ins(4,5,6)P_3, will also yield xylitol, but in so doing the 2-carbon with its attached hydroxyl and hydrogen will be lost, eventually to be evaporated off as methanol. As the radiolabel of the original *myo*-[^{3}H]inositol is in the 2 position, the > 80% conversion of [^{3}H]InsP_3 to [^{3}H]xylitol in these experiments eliminates Ins(4,5,6)P_3 as a possibility.]

The InsP_4 must therefore, in view of its resistance to periodate, have its fourth phosphate in the D- or L-(1) position. This was confirmed independently by the observation that human erythrocyte ghosts could quantitatively convert the InsP_4 to InsP_3 with < 5% formation of InsP_2, Ins$P$ or inositol. On periodate, borohydride and alkaline phosphatase treatment, this InsP_3 yielded labelled altritol with 85% recovery, which is indicative of a D-or L-Ins(1,2,4) or Ins(1,3,4) structure (Irvine *et al.*, 1984*a*). As with the xylitol (see above), ionophoretic separations of differing length were performed to ensure both the identification of altritol, and that it was the only alditol formed. The InsP_3 under examination also co-chromatographed exactly with Ins(1,3,4)P_3 on h.p.l.c. (Irvine *et al.*, 1985) and, in view of the observations above with regard to the (3,4,5) grouping in the InsP_4, it must therefore be D- or L-Ins(1,3,4)P_3. As well as confirming independently that the InsP_4 has a phosphate in the D- or L- (1) and (4) positions, these experiments reveal that human red cells have an enzyme(s) which in addition to specifically removing the 5-phosphate from Ins(1,4,5)P_3 (Downes *et al.*, 1982) also removes the 5-phosphate from InsP_4 to form Ins(1,3,4)P_3, a compound which is formed in several

stimulated tissues (Irvine *et al.*, 1984; Burgess *et al.*, 1985) including brain (Fig. 1).

Source of Ins(1,3,4,5)P_4

The most likely source of Ins(1,3,4,5)P_4 is phosphodiesteratic hydrolysis of a PtdIns(3,4,5)P_3. Evidence for PtdInsP_3 has been reported previously (Klenk & Hendricks, 1961; Santiago-Calvo *et al.*, 1963), and though further observations suggested that at least the first of these might have been due to phosphate contamination (Kerr *et al.*, 1963), the evidence of Santiago-Calvo *et al.* (1963) suggests that PtdInsP_3 may exist in brain, albeit at low levels (Seiffert & Agranoff, 1965). We looked for [^{3}H]PtdInsP_3 in lipids extracted by acidified chloroform/methanol from the trichloroacetic acid precipitates in some of the present brain slice experiments, using methods similar to those by which we sought PtdIns(3,4)P_2 in parotid glands (Irvine *et al.*, 1984*a*) [i.e. a chemical degradation of the lipids to inositol phosphates using deacylation (Clarke & Dawson, 1981) and removal of the glycerol moiety (Brown & Stewart, 1966) followed by examination of the resulting inositol phosphates by h.p.l.c.]; we were unable to detect any InsP_4 in these experiments, and we can say that in our extracts PtdInsP_3 must be less than 1% of the PtdIns(4,5)P_2 radioactivity. We have, however, no guarantee that the extraction method we used would be effective for recovering PtdInsP_3. Furthermore, a very rapidly turning over pool of PtdInsP_3 could be difficult to detect, yet be able to generate the levels of InsP_4 that we measure. The only obvious alternative route of synthesis of InsP_4 would be by a phosphorylation of Ins(1,4,5)P_3, but we think that a lipid precursor [i.e. a phosphorylation of PtdIns(4,5)P_2 on the 3-hydroxyl] is more likely. Either way, in view of the known structure of PtdIns(4,5)P_2 as the D-isomer (Grado & Ballou, 1961; Brown & Stewart, 1966; Agranoff, 1978), it is probable that the InsP_4 under study is D-*myo*-inositol(1,3,4,5)P_4.

DISCUSSION

Our identification here of Ins(1,3,4,5)P_4, as with the previous identification of Ins(1,3,4)P_3 in parotid glands (Irvine *et al.*, 1984*a*), can only be preliminary in that it is based on indirect methods centered on co-chromatography of radioactivity with authentic standards. Nevertheless, within the limits of such methods, the only structure consistent with all the data is Ins(1,3,4,5)P_4, most likely the D-isomer. The rapid formation of Ins(1,3,4,5)P_4 in stimulated brain slices suggests the possibility that, like Ins(1,4,5)P_3 (Berridge & Irvine, 1984), it has second messenger functions.

The most obvious function would be as an adjunct to Ins(1,4,5)P_3 in calcium mobilization, or even as a more important message in this respect. However, as the metabolism of Ins(1,4,5)P_3 in stimulated tissues is itself so strongly consistent with its proposed second messenger role (Berridge *et al.*, 1984; Irvine *et al.*, 1985), and also as Ins(1,4,5)P_3 is such a potent and specific mobilizer of calcium in permeabilized cells (Streb *et al.*, 1983; Irvine *et al.*, 1984*b*), a completely separate messenger function of Ins(1,3,4,5)P_4 must be regarded as at least as likely a possibility.

One thing we can say with some degree of confidence is that Ins(1,3,4,5)P_4 is the probable precursor of Ins(1,3,4)P_3. We cannot be sure whether the same enzyme in red cell membranes as that which hydrolyses Ins(1,4,5)P_3 (Downes *et al.*, 1982), is responsible for Ins(1,3,4,5)P_4 hydrolysis, and this question must await purification of the enzyme(s). But the remarkably specific removal of the 5-phosphate from Ins(1,3,4,5)P_4 to form Ins(1,3,4)P_3 in these experiments shows that such an enzyme does exist in at least one tissue, and this, allied with our former inability to detect PtdIns(3,4)P_3 (Irvine *et al.*, 1985; Burgess *et al.*, 1985) or an Ins(1,4,5)P_3 isomerase (R. F. Irvine & M. J. Berridge, unpublished work), makes the conclusion almost inescapable that Ins(1,3,4)P_3 is the normal enzymic breakdown product of Ins(1,3,4,5)P_4. Finally, it should be considered that if Ins(1,3,4,5)P_4 is derived from a lipid then it could also be the precursor of Ins(1,4,5)P_3, with the proportion of the InsP_3 isomers generated dependent on the respective activities of appropriate 5- and 3-phosphatases. Such speculation, as well as the intriguing possibility that the hydrolysis of PtdIns(3,4,5)P_3 could be the event directly controlled by receptors, can only be tested by further experimentation. Whatever the function or catabolism of InsP_4, the data here open up a new facet, and a new pathway, of inositide metabolism which is an exciting area for experimental exploration.

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